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Article

Cellular and subcellular effects of chronic low-dose Lambda-cyhalothrin pesticide exposure modulated by medicinal plant methanol extract in rat

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Abstract: The extensive use of Lambda-cyhalothrin (LCT) has been associated with the various toxicities that non-target organisms can undergo including mammals. However, the mechanism of LCT-induced cytotoxicity in animal brain cells is still elusive, particularly in brain regions, notably the hippocampus, an area directly involved in cognitive function. This study aimed to investigate the neurotoxic effects in the rat hippocampus chronically exposed to LCT (0.18 mg/kg and 0.36 mg/kg), and the neuroprotective potential of *Melissa officinalis* L methanol extract (MOE) (200 mg/kg) against this toxicity. After experimental period (90 days), the redox status, the functional and structural integrity of the hippocampus mitochondria as well as the apoptotic signaling pathway were evaluated. The current findings suggest that LCT induces an imbalance of mitochondrial redox status characterized by, on one side, an increase of stress markers such as protein carbonyls (PCO), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) levels; and on the other side, a decline in the potential of antioxidant systems, namely the level of mitochondrial enzymatic activities of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and glutathione (GSH).This study also showed an increase in mitochondrial permeability, along with mitochondrial edema and considerable decrease in its O_2 consumption. Moreover, the same results recorded an increase in caspase-3 and cytosolic cytochrome-c. Conversely, this study proved that all these toxic aspects induced by LCT were significantly mitigated when the administration of this synthetic pyrethroid was associated with MOE. Taken together, data of this study shed light on mitochondrial damage and apoptosis stimulation under the toxic effect of LCT and suggests that MOE is endowed with potent neuroprotective effects, possibly via its antioxidant and antiapoptotic properties.

Keywords: Lambda-cyhalothrin; *Melissa officinalis* L; hippocampus; stress markers; mitochondrial edema; apoptosis; caspase-3; cytochrome-c; antioxidants

1. Introduction

Phytosanitary products play an essential role in agriculture; by protecting crops against pests, diseases, weeds, and insect pests [1]. Insecticides represent nearly a quarter of the phytosanitary product market [2]. However, it is expected that extensive use of these chemicals leads to the contamination of foods by their residues which presents a hazardous risk for non-target organisms, in particular, for human health [3]. Indeed, these chemicals are responsible for the appearance of several types of diseases in humans and animals, such as hematologic disorders [4], cancer and reproductive dysfunction [5], endocrine disruption [6], immunomodulation [7] and neurodegenerative and neurobehavioral disorders [8]. A

new generation of pyrethroids (Deltamethrin and lambda-cyhalothrin) are widely used in agriculture in Algeria because they are practical and more water-soluble, thus less persistent, compared to organochlorines and organophosphates. Although their insecticidal action is well studied, understanding synthetic pyrethroids fate in biological matrices and their degree of toxicity has become a concern in the world's developed countries [9]. Extensive use of LCT has been associated with serious potentially adverse effects to the non-target organisms including humans and animals. Indeed, LCT has several toxic effects, given that it acts in more several enzyme pathways, it has also a great potential to trigger oxidative stress, disturb neurotransmitter activities and modulate immunity [10,11]. More specifically, according to the work of Ansari et al. [12], exposure of rats to this non-systemic pesticide at the dose of 0.5 mg/kg body weight, p.o., during 28 days, showed no effect in lipid peroxidation and protein carbonyl levels in frontal cortex, corpus striatum, hippocampus and hypothalamus, while de study developed by Bush et al. [13], indicated that 28 days oral exposure of LCT causes oxidative damage to brain of mice which in turn could be responsible for neurotoxicity. Just after, another study suggested that induction of oxidative stress and DNA damage were associated with histological changes in the brain of exposed rats to one third LD_{50} [14,15]. It should be mentioned that, according to the literature consulted, the studies carried out on this non-systemic pesticide at regional level of the brain, especially hippocampus area, are not sufficient and often lack the mechanisms of underlying effect of LCT on animal neurotoxicity. Nevertheless, further detailed studies are required to prove these effects mainly after long-term exposure. Hippocampus is a complex structural entity deeply embodied into temporal lobe of the brain and forms part of a limbic system. It is associated principally with learning and memory [16]. This brain area is plastic and vulnerable structure which can be damaged by synthetic pyrethroids [17]. Several neurodegenerative diseases are issued by deleterious effect of free radicals as they are responsible for many kinds of neurodegenerative diseases which are the causes of locomotive disorders (Parkinson's disease) and loss of memory and ability to learn (Alzheimer's disease) [8,18]. In this pathological context, scientific attention is focused on the cytoprotective potential of nutraceutical or bioactive phytosubstances produced by medicinal plants. Indeed, herbal remedies with neurobiological activity may be potential targets for drug discovery [19]. Their potent bioactivities and relatively low toxicities have provided them with the right ingredients in complementary alternative medicine and nutritional supplementation [20]. According to previous studies, this plant was used in herbal medicine as a sedative for the prevention and treatment of sleep disorders [21,22] and also as cardioprotective against ischemia and heart injury [23]. Furthermore, several studies showed that the ethanolic extract of *Melissa officinalis* L. considerably improves learning and memory in rats [24,25]. Some work has also reported that *Melissa officinalis* is provided with antioxidant effects, thus, it can be concluded that it has therapeutic and preventive potential against oxidative stress-related diseases [23]. Nevertheless, according to current scientific data, it seems that there is no attention paid to experiment *Melissa officinalis* L effect versus the neurotoxicity of the pesticides in long-term LCT exposure. Therefore, this study aimed to evaluate LCT potentially toxic effects on the hippocampus and identify the connection between the

various oxidative stress mechanisms and the relevance of disruption of mitochondrial function and integrity for apoptotic triggering; and also, to seek potential role in barrier cytoprotection of *Melissa officinalis* L.

2. Materials and methods

2.1. Chemicals

Lambda-cyhalothrin is a synthetic pyrethroid insecticide ($C_{23}H_{19}ClF_3NO_3$); CAS chemical name (a-cyano-3-pfenoxybenzyl-3-(2-chloro-3,3,3-tripfluoro-1-propenyl); CAS registry number (91465-08-6). The experiment used a commercial LCT formula called the KARATE® product (Syngenta Agrochemicals, Greensboro, USA). Almost all chemicals were purchased from Sigma Aldrich, Germany. Assay Kits for enzymes were purchased from Biomerieux, France. Caspases substrates were purchased from Beckman, USA, and ELISA kits from Uscn Life Science Inc. China, BCA Protein Assay Kit (Thermo Scientific, Rockford, IL. USA).

2.2. Plant material and preparation of *Melissa officinalis* L methanol extract (MOE)

In this study, *Melissa officinalis* L leaves were harvested in October 2018 from Tebessa, South-East of Algeria. To avoid the decomposition of some bioactive compounds, the samples were packed instantly in polyethylene bags containing ice and transported to the laboratory within 2h. The MOE was prepared following Bruneton recommendations [26]: briefly, 100 g of leaves were extracted by maceration in 1000 mL of methanol-water (7:3, v/v) with agitation for 24 h at 200 rpm. After that, the methanol-soluble fraction was filtered and concentrated under reduced pressure at 40 °C using a rotary evaporator. For long-term conservation, the extract is lyophilized and stored in the dark at 4 °C. The extraction yield was 13.37% (w/w).

2.3. Animal treatments

Male rats were obtained from the Pasteur Institute in Algiers, Algeria (PIA), and weighing 220–260 g at approximately 8–12 weeks of age. Upon arrival, the animals were housed 6 per cage with food and water ad libitum. Animals were maintained under a daily 12 h light/dark cycle at a constant temperature (24 ± 4 °C), relative high humidity of 60% All experiments were carried out according to International Guidebook on Treatment, and Use of Laboratory. The animals have been divided into 6 groups of six male rats each. Every week, a sufficient amount of the powdered extract is dissolved in corn oil, the vehicle. Treatment was carried out using a probe attached to the syringe for daily oral gavage of the prepared solutions during 90 days continuously. The different groups were distributed as follows:

- Group1: Control (CT), received the vehicle only;
- Group2: MOE, received 200 mg/kg of extract [24];
- Group3: LCTD1, received D1 = 0.18 mg/kg/day of LCT;
- Group4: LCTD2, received D2 = 0.36 mg/kg/day of LCT;

- Group5: LCTD1 + MOE; received D1 = 0.18 mg/kg/day of LCT + 200 mg/kg of extract;
- Group6: LCTD2 + MOE; received D2 = 0.36 mg/kg/day of LCT + 200 mg/kg of extract.

Each rat receives an amount of treatment solution equal to 1 mL per 250 g of body weight.

2.4. Extraction of hippocampus mitochondrial matrix

After 90 days of treatment, animals were sacrificed by decapitation after prolonged deep ether anesthesia. The brain was retrieved, immediately washed with cold phosphate-bumped saline (PBS); the hippocampus was immediately separated, which is regarded as the source of fresh mitochondria suspension, mitochondrial matrix and cell lysates to evaluate LCT and MOE effects on permeability and mitochondrial edema, mitochondrial respiration, mitochondrial redox status and apoptosis markers. According to the method described by Chouit et al. [27], the entire mitochondrial fractions were freshly extracted at the end of the treatment period. To recover the mitochondrial matrix, we proceeded to freezing and thawing associated with repeated homogenization, approximately eight times to burst the mitochondrial membranes, then we carried out centrifugation 10.000 g for 10 min. The obtained supernatant was used as a source of samples for assays of different oxidative stress parameters (MDA, PCO, H_2O_2, GSH, CAT, GPx, and SOD).

2.5. Evaluation of swelling, permeability and mitochondrial respiration

Mitochondrial membrane permeability was rapidly evaluated with fresh tissue of the studied hippocampus according to the method described by Li et al. [28]. Calcium treatment can cause mitochondrial swelling which has been used as an indicator of increased opening of mitochondrial permeability transition pore (mPTP). Briefly, the isolated mitochondrial pellet step was resuspended in an assay buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris-Base, Pi(K) 100 mM, 0.5 mM Mg_2, pH 7.0). A mitochondrial aliquot (100 mg) was diluted at 25 ℃ in 1 mL of assay buffer. To induce swelling, 200 mM $CaCl_2$ were added and the aliquot was analyzed by spectrophotometer as a decrease in light dispersion for 5 min at 540 nm. To calibrate the light transmission propofol at 200 mM was added to the mitochondrial suspension 1 min before addition of $CaCl_2$. The experiment was conducted using an assay buffer.

Mitochondrial respiration was estimated using an Oxygraph (HansaTech) according to the method described by Ortiz-Jiménez et al. [29]. Briefly, after calibration of the Oxygraph, pellets were resuspended in a final volume of 0.5 mL into the Oxygraphs water-jacketed chamber containing PTA buffer with 10 mM sodium succinate, 0.01% digitonin and 200 mM ADP. Using the equipment's plug, the chamber was closed while making sure no air bubbles remain inside. After 5 min, oxygen consumption rate was measured for 1–2 min (state 3). 2 mg oligomycin were injected and oxygen consumption rate measured again for 1–2 min (state 4). Respiratory Control Ratio (RCR) was calculated by dividing State 3 by State 4 which considered O_2 consumption.

2.6. Preparation of hippocampus lysates

Cell lysates were extracted hippocampus according to the method of Ahmed et al. [30]. In 1 mL lysis buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, and 1.0 mM DTT) with protease inhibitors (2 µg each of aprotinin, leupeptin, pep statin A, and 0.5 mM phenylmethylsulphonyl fluoride), homogenize minced tissues of hippocampus (0.5 g). Incubate on ice for 30 min, and then centrifuge at 10,000 × g at 4 ℃ for 20 min. Apoptosis markers were evaluated on separated hippocampus lysates.

2.7. Biochemical evaluation of hippocampus mitochondrial oxidative stress

Matrix MDA concentration was measured based on the method of Esterbauer [31]. Protein carbonyls (PCO) content was quantified by the reaction with 2,4-dinitrophenylhydrazine (DNPH), as reported by Reznick and Packer [32]. Hydrogen peroxide (H_2O_2) generation was assessed by Ou and Wolff method [33]. The GSH content was determined according to the method of Ellman [34], modified by Davis et al. [35]. Total superoxide dismutase activity (SOD) was evaluated by measuring pyrogallol activity inhibition, as described by Marklund and Marklund [36]. CAT activity was assessed according to the method of Aebi [37]. Glutathione peroxidase (GPx) activity was measured according to Gonzler method [38].

2.8. Determination of cytochrome-c and caspase-3 activities

Determination of cytochrome-c (Cyt-c) and caspases-3 activities in the hippocampus tissue lysates was performed using a commercial kit (Caspase-3 Colorimetric Activity Assay Kits, part no. 90079, (Sigma®)). The Cyt-cAssay Kit (CYTOCOX1) uses an optimized colorimetric assay based on observation of the decrease in absorbance of ferro-Cyt-c-c measured at 550 nm. Levels were expressed as units per milligram of proteins.

2.9. Assessment of acetylcholinesterase activity (AChE) and proteins in tissue lysates

Acetylcholinesterase (AChE) activity was determined, after hydrolysis of AChE, according to Ellman et al.'s method [39]. The results were expressed as µmol per milligram (mg) of proteins (P) per min (µmol/min/mg P), while the evaluation of the proteins was measured by Bradford method [40], using bovine serum albumin as the standard.

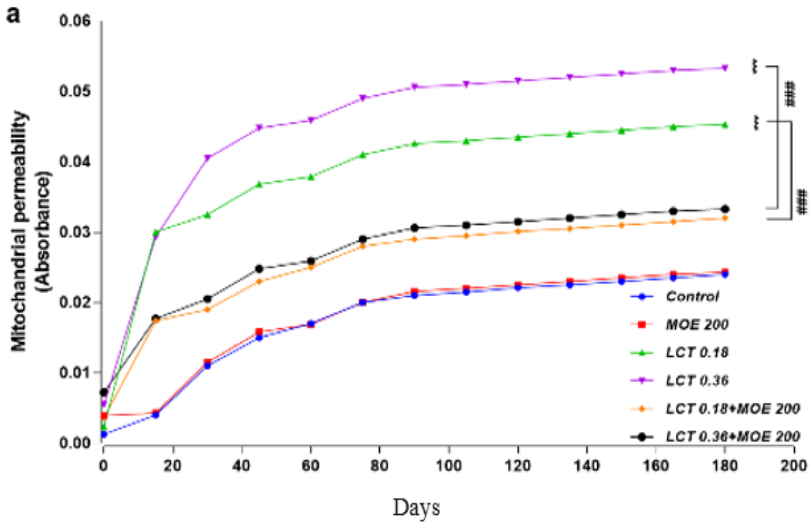
2.10. Statistical analysis

Results were expressed as mean ± standard deviation (mean ± SD). The analysis was carried out with GraphPad Prism 8.0 for Windows (GraphPad Software, San Diego, CA). Significant differences between treatment effects were determined by one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons with statistical significance of $p < 0.05$.

3. Results

3.1. Measurement of permeability, swelling, and mitochondrial respiration

The changes of mitochondrial parameters (permeability, swelling, and respiration) of rats treated with LCT at both doses LCT 0.18; and LCT 0.36 (mg/kg); *Melissa officinalis* L extract alone (MOE; 200 mg/kg/day) and their combination (MOE + LCT 0.18, and MOE + LCT 0.18) for 90 days are presented in **Figure 1**. In the hippocampus mitochondria, there was an increase in ($p < 0.001$) mitochondrial permeability in rats (**Figure 1a**), treated with LCT 0.18. LCT 0.36 (mg/kg) in comparison with the control, although these findings indicate substantial difference ($p < 0.001$) in rats treated with combination (MOE + LCT 0.18, and MOE + LCT 0.36) compared to groups LCT 0.18; and LCT 0.36 (mg/kg) respectively. The results in (**Figure 1c**) show a significant augmentation ($p < 0.001$) of mitochondrial swelling in hippocampus of rats treated with LCT 0.18; and LCT 0.36 (mg/kg) compared to the control, while after additional MOE, this result is neutralized in MOE + LCT 0.18 mg/kg, and MOE + LCT 0.36 mg/kg) compared to groups LCT 0.18 ($p < 0.001$); and LCT 0.36 ($p < 0.01$) (mg/kg) respectively. Treatment of rats by LCT 0.18 and LCT 0.36 (mg/kg) decreases mitochondrial respiration (**Figure 1b**), but after MOE's association with pesticide, this decrease is normalized.



(a)

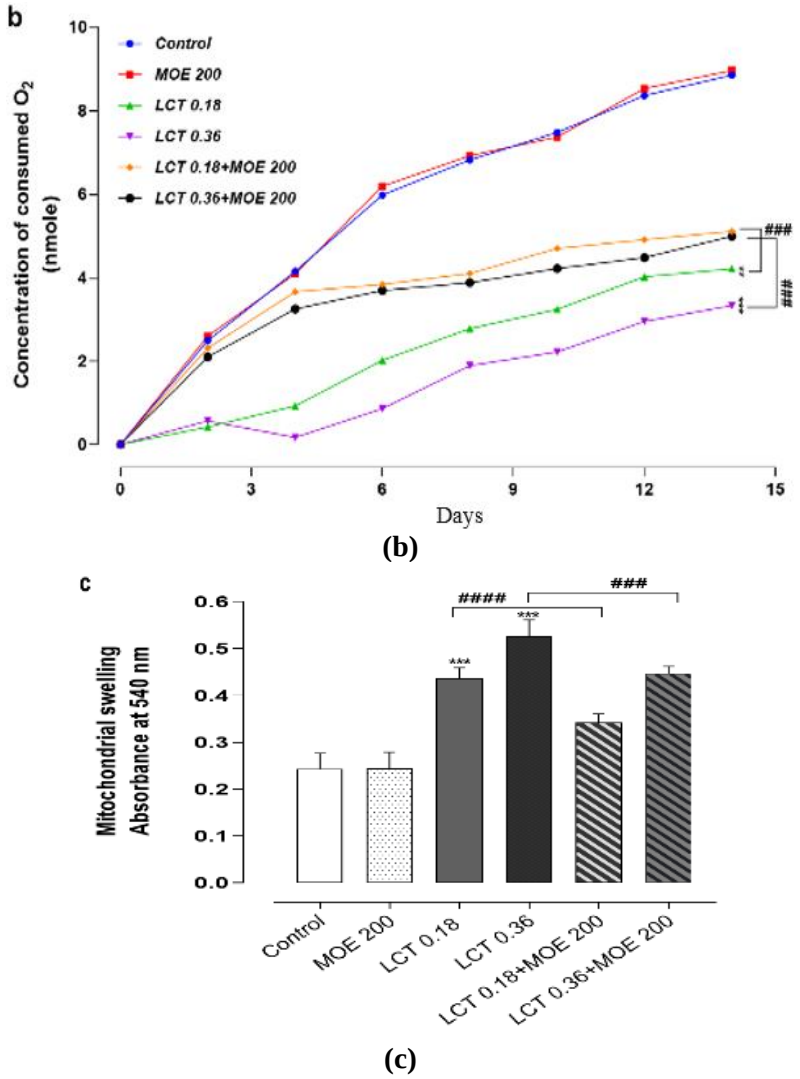


Figure 1. Effect of oral administration of LCT at different doses 0.18 and 0.36 (mg/kg), *Melissa officinalis* L extract (MOE) alone (MOE 200 mg/kg) and their combination (MOE + LCT 0.18, and MOE + LCT 0.36) for 90 days on **(a)** mitochondrial permeability; **(b)** mitochondrial respiration; **(c)** mitochondrial swelling.

N = 6 rats/group. Results in each group represent mean ± SEM.; *** $p < 0.001$: LCT-treated group compared with control group; ### p: < 0.001 (MOE + LCT 0.18 and MOE + LCT 0.36) groups compared with LCT 0.18; and LCT 0.36 (mg/kg) groups.

3.2. LCT and MEO exposure effects on oxidative stress markers

Protein carbonylation (PCO), lipid peroxidation (MDA), and hydroperoxide generation (H_2O_2) in hippocampus mitochondria of treated rats with LCT at both doses (0.18 mg/kg, 0.36 mg/kg); MOE alone (200 mg/kg/day) and their combination (MOE + LCT) for 90 days are presented in **Figure 2a–c** respectively. Statistical study showed a significant increase in PCO ($p < 0.05$; $p < 0.001$), MDA ($p < 0.001$) and H_2O_2 levels ($p < 0.001$) of treated-LCT group in comparison with the control group. The administration of MOE 200 mg/kg associated with both doses of LCT (0.18, and 0.36 mg/kg), showed a significant ($p < 0.01$) reduction of MDA and non-significant lowering for PCO level compared to LCT-treated groups. Otherwise, the administration of MOE associated with LCT 0.36 (mg/kg), revealed a significant

reduction ($p < 0.01$) compared to LCT group receiving the dose of 0.36 (mg/kg), but this reduction was not observed when MOE was administered with LCT at the dose of 0.18 (mg/kg) compared to group LCT 0.18 (mg/kg).

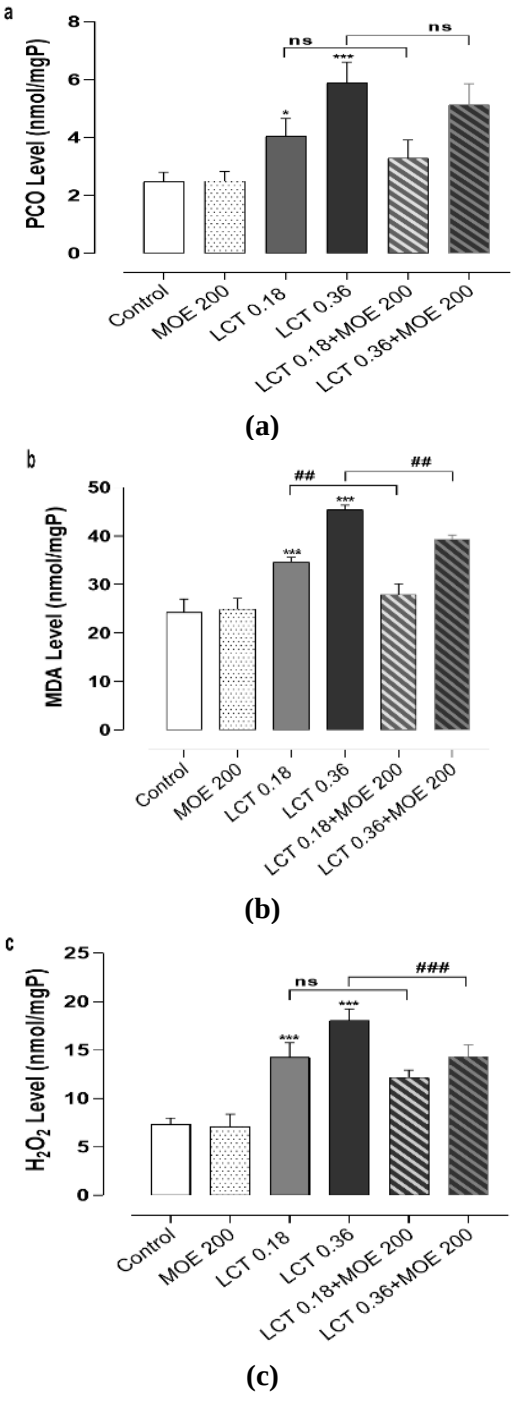
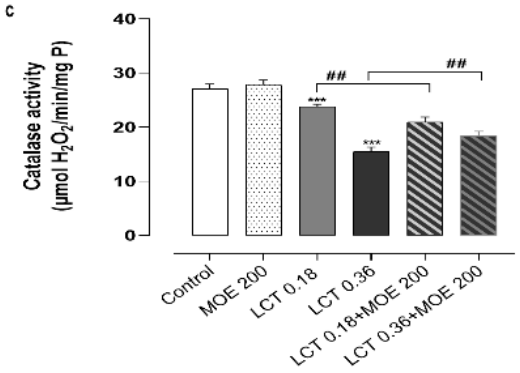


Figure 2. Effect of oral administration of LCT at different doses 0.18; and 0.36 (mg/kg/day), *Melissa officinalis* L extract (MOE) alone (200 mg/kg/day) and their combination (MOE + LCT 0.18 and MOE + LCT 0.36) for 90 days **(a)** protein carbonylation level (PCO); **(b)** lipid peroxidation level (MDA); **(c)** hydroperoxide generation (H_2O_2).

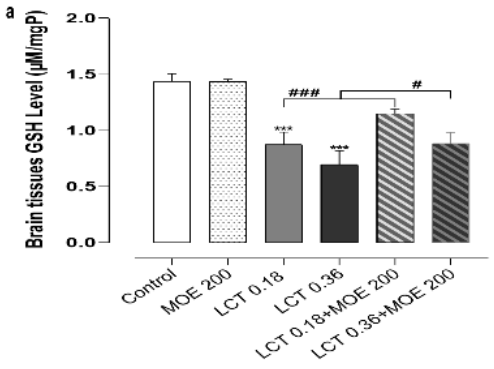
N = 6 rats/group. Results in each group represent mean ± SEM.; * $p < 0.05$, *** $p < 0.001$: LCT-treated group compared with control group; ns: no significant (MOE + LCT 0.18, and MOE + LCT 0.36) groups compared with LCT 0.18 and LCT 0.36 (mg/kg/day) groups.

3.3. LCT and MOE exposure effects on antioxidant defense System

Glutathione (GSH), Superoxide dismutase (SOD), catalase (CAT) and Glutathione peroxidase (GPx) activities of rats treated with LCT at both doses 0.18 and 0.36 (mg/kg); *Melissa officinalis* L extract (MOE) alone (200 mg/kg/day) and its combinations (MOE + LCT 0.18, and MOE + LCT 0.18) for 90 days are shown in **Figure 3a–d** respectively. One-way ANOVA recorded that the antioxidant enzyme systems in hippocampus mitochondria have significant difference. Indeed, statistical analysis has showed that LCT) at both doses (0.18 and 0.36 (mg/kg) caused a significant decrease in GSH activity ($p < 0.001$), SOD activity ($p < 0.05$; $p < 0.01$) respectively, in CAT activity ($p < 0.001$, $p < 0.001$) respectively and in GPx activity ($p < 0.05$; $p < 0.001$) in comparison with the control group. The administration of MOE at 200 (mg/kg), associated with 0.18 and 0.36 (mg/kg), increased significantly this tripeptide (GSH) in comparison to 0.18 ($p < 0.001$), and 0.36 ($p < 0.05$) (mg/kg) groups, at the both doses (0.18 and 0.36 mg/kg) improved significantly ($p < 0.01$) the activity of all enzymes studied (SOD, CAT, and GPx) compared to LCT-treated groups.



(a)



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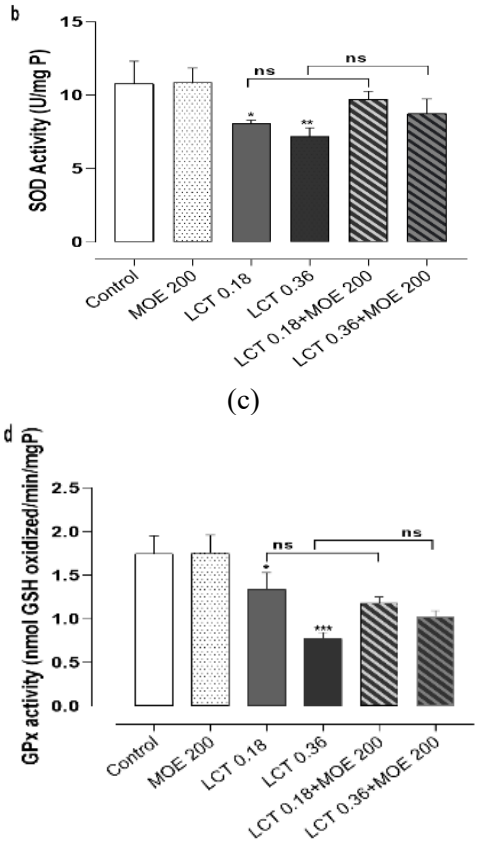

Figure 3. Effect of oral administration of LCT at different doses 0.18 and 0.36 (mg/kg/day), *Melissa officinalis* L extract (MOE) alone (200 mg/kg/day) and their combination (MOE + LCT 0.18 and MOE + LCT 0.36) for 90 days **(a)** Glutathione (GSH) activity; **(b)** superoxide dismutase (SOD) activity; **(c)** catalase (CAT) activity; **(d)** glutathione peroxidase (GPx) activity.

N = 6 rats/group. Results in each group represent mean ± SEM.; *** $p < 0.001$: LCT-treated groups compared with control group; # $p < 0.05$, ### $p < 0.001$: (MOE + LCT 0.18 and MOE + LCT 0.36) groups compared with LCT 0.18 and LCT 0.36 (mg/kg/day) groups.

3.4. LCT and MEO exposure effects on Cyt-c and caspase-3 activities

Figure 4a,b show respectively Cyt-c and caspase-3activities in hippocampus tissue lysates of rats treated with LCT at the both doses (0.18 and 0.36 (mg/kg), MOE alone (200 mg/kg/day) and its combinations (MOE + LCT 0.18 and MOE + LCT 0.18) for 90 days. One-way ANOVA analysis showed significant increase ($p < 0.001$) in both Cyt-c and caspase-3 activities in LCT-treated groups compared to the control group. Also, it was demonstrated that administration of MOE associated with both LCT doses significantly decreased the relative caspase-3 and Cyt-c activities in hippocampus tissue when compared to LCT-treated groups. However, the administration alone of MOE 200 mg/kg/day) did not affect Cyt-c and caspase-3 activities in hippocampus tissue lysates.

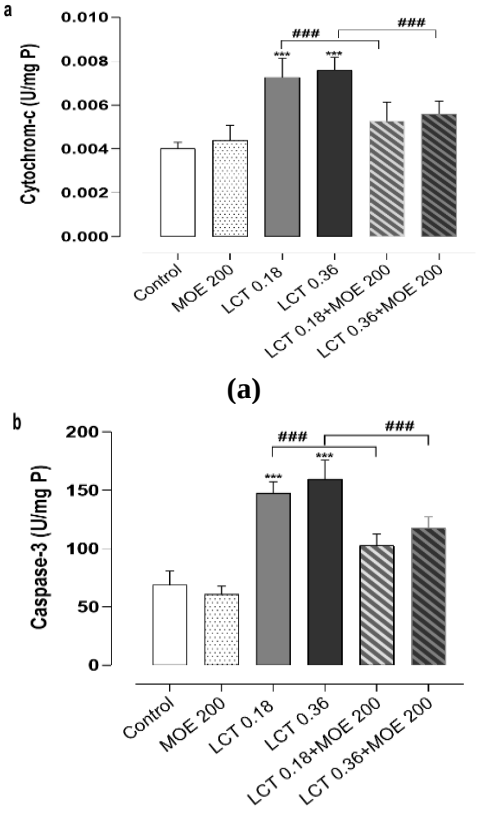

Figure 4. Effect of oral administration of LCT at the doses of (0.18 and 0.36 mg/kg/day), *Melissa officinalis* L extract (MOE) alone (200 mg/kg/day) and its combinations (MOE + LCT 0.18 and MOE + LCT 0.36) for 90 days on **(a)** cytochrome-c activity; **(b)** caspase-3 activity in hippocampus tissue lysate.

Results in each group represent mean ± SEM, (N = 6); *** $p < 0.001$: LCT-treated groups compared with Control group; ### $p < 0.001$: (MOE + LCT 0.18, and MOE + LCT 0.36) groups compared with LCT-treated groups.

3.5. LCT and MOE exposure effects on AchE activity

As shown in **Figure 5**, a significant decrease in activity of AchE ($p < 0.001$), in hippocampus tissue was observed in LCT-treated groups compared to control. But administration of MOE at 200mg/kg associated with both doses used of LCT (0.18 and 0.36 mg/kg), did not change significantly the activity of this enzyme in comparison with LCT-treated groups.

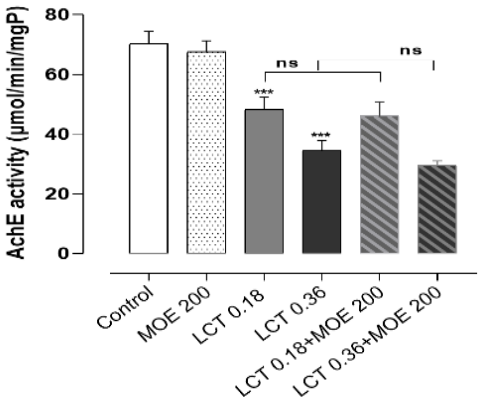

Figure 5. Variation of AchE activity under the effect of LCT at two doses (0.18 and 0.36 (mg/kg/day), *Melissa officinalis* L extract (MOE) alone (200 mg/kg/day) and its combinations (MOE + LCT 0.18 and MOE + LCT 0.18) for 90 days.

Results represent mean ± SEM, $N = 6$. *** $p < 0.001$, LCT-treated groups compared with control group; ns: no significant (MOE + LCT)—treated groups compared with LCT-treated groups.

4. Discussion

The brain is confronted with lesions associated with free radicals due to its high demand for energy but also because of the weakness of its antioxidant systems such as SOD, CAT and vit-C and the imbalances often occurring in polyunsaturated AGs, proteins and nucleic acids [41]. This brain vulnerability to radicals, reactive oxygen species (ROS) can simply modify proteins and enzymes for cytoarchitecture disintegration [41,42]. In this context, brain enzyme systems are enabled to produce autolytic characteristics for the cell, especially the lipolysis process, proteolysis and protein phosphorylation [43,44]. In the current research, after 90 days of LCT exposure in rats, a significant increase was shown in mitochondrial permeability and swelling. Recent studies have discussed dilation of the endoplasmic reticulum and a break in the membrane after an increase in the volume of the cell and the mitochondrial swelling after a large calcium and water inflow [7,8]. Mitochondria permeabilization not only causes mitochondria edema but eventually provokes a release of several mitochondrial intermembrane pro-apoptotic proteins in the cytosol such as Cyt-c, a key step in the initiation of apoptosis to cause cell death [45]. However, this loss of membrane integrity often occurs when mitochondrial breathing is decreased, and O_2 is consumed in small amounts [46]. In fact, the current study showed a significant decrease in respiration rate of mitochondria in LCT-treated rats, justified therefore by redaction of consumed O_2 in LCT-treated rats. Previous research regarding other pesticides, showed a strong association of ROS production with inhibition of mitochondrial respiration [47]. In this study, the results showed that hippocampus mitochondria extracted from LCT-treated rats did not respire well in comparison with control and MOE-LTC-treated groups; furthermore, these organelles produced a high level of oxidative stress markers. While this discovery did not provide specific information for cytochrome complexes in electron transport chain, but it did suggest that LCT could potentially inhibit mitochondria functions in the rats' hippocampus. Some recent studies have suggested that insecticides lead to neurotoxicity which could be induced by oxidative stress [45,46]. The results of this

study showed a significant rise in the MDA and PCO levels in rat hippocampus in LTC-treated animals. These data indicate that LCT triggered free radicals in the hippocampus mitochondria at both doses of LCT. The same results were obtained in a previous study, since there was a correlation between exposure to LCT exposure and ROS effects in rats [47–49]. In this study, the relative values of anti-oxidative systems such as SOD, CAT, GPx and GSH decreased significantly in hippocampus mitochondria of LCT-treated rats. Several researchers have confirmed these findings when pyrethroid insecticides were administered acutely or chronically to the rats [14,48,49]. The brain's vulnerability to ROS generation and the loss of antioxidant potential is supported by the fact that the brain consumes high amounts of oxygen and mainly due to its richness of polyunsaturated fatty acids which could be targeted by ROS attack which produces intense lipid peroxidation and thus disturbs neuron membrane structure [17,27,50]. It is well known that H_2O_2 is the product of the dismutation of O_2—by superoxide dismutase and on the other side it is the substrate of CAT which transforms it into H_2O and O_2. In the present study, the obtained results showed, in addition to the loss of antioxidant systems, a high increase in mitochondrial H_2O_2, MDA and PCO. H_2O_2 is expected to increase substantially in the mitochondrial matrix as long as the activity of enzymes using the peroxide as substrate especially Cat and GPx. In fact, several previous research works showed an increase in H_2O_2 at the same time of the collapse of the enzymatic activity of SOD, CAT and GPx in brain mitochondria of the rats exposed to pesticides [51–53]. The results of previous studies have shown the involvement of ROS indirectly by reaction with reactive carbonyl species derived from the lipid peroxidation such as MDA in severe oxidative stress [54]. These carbonylated proteins, instead of being degraded by proteasome, can escape and aggregate to deteriorate the cell and its organelles [55]. Protein carbonylation has attracted research attention because carbonylated proteins have irreversible and unrepairable properties presenting a powerful molecular etiology in many degenerative diseases [54,55]. In the current study, the results showed a substantial increase in PCO at the same time as an increase in mitochondrial MDA (PCO-maker) of rat striatum in LCT-treated rats, which provides information on the molecular and functional lesions of the mitochondrial proteome. Mitochondrial alterations concerning respiration function and ATP synthesis lead to mitochondrial membrane depolarization and later voltage-gated receptor activation, permitting influx of calcium in the cytosol and triggering cell death [56,57]. Indeed, mitochondria are the hub of redox status activity and cellular stress control, so it turns out to be the most important organelle in switching cell survival and are considered safety device against toxic elevation increase of cytosolic rate of Ca^{++}, where the permeability transition pore of their membranes could be a critical point in cell death [58]. In light of the arguments and views in this debate, the mitochondrial edema, membrane permeability imbalance and the fall of mitochondrial respiratory function that we reported in the results of this study in LCT-treated rats, may originate in the imbalance of redox status triggered by the pesticide for the benefit of ROS and pro-oxidant substances. According to literature, these mitochondrial abnormalities lead generally to cytochrome-c release into cytosol, which participates in caspase-9 maturation required for the activation of apoptosis pathway signaling [8,27]. Indeed, the results

of this study revealed an increase in cytosolic Cyt-c and caspase-3 in LCT-treated rats that is considered the logical implication of mitochondrial functions collapse and the loss of its membrane integrity. Numerous researchers reported that the induction pathway of the predominant apoptosis depends on mitochondria when exposed to a pesticide [54,55] and the current study supports also the hypothesis that in apoptosis's implementation process, caspases sequential activation plays a central role [43,59]. The scheme consists of releasing apoptogenic proteins into the cytosol from mitochondrial intermembrane space, such as apoptotic-inducing factor (AIF) and Cyt-c, and if the cytoplasm is extended, the latter results in apoptotic peptidase activating factor 1 (Apaf-1) and caspase-9 recruitment leading to apoptosome formation which triggers the intrinsic or apoptosis mitochondrial pathway [60,61]. In this case, procaspase-9, an initiator caspase in the intrinsic apoptosis signaling, is recruited and activated by the apoptosome leading to downstream caspase-3 processing [62,63]. Moreover, in this study, Chronic LCT Exposure showed a significant decrease in AChE activity, knowing that this enzyme is important in developing rats and memory and learning functions [64]. These findings are consistent with Ansari et al.'s earlier research which showed reduced AChE activity in rat hippocampus exposed to LCT and bifenthrin [12,46], and it has been reported that the decrease in this activity is due to blockage of AChE catalytic site [12]. In this study, the administration of Melissa officinalis L. extract at the dose of 200 mg/kg/day for 90 days, restored the levels of GSH, SOD, CAT, and GPx of LCT-exposed rats, which decreased ROS concentrations by scavenging the ROS directly [65] or by transcriptional enhancement of antioxidant genes [66]. These results support the hypothesis that intrinsic role of MOE as an antioxidant, could neutralize reactive species, and/or its ability to enhancing the hippocampus's antioxidant protection mechanism. These findings suggest that MOE protective action is not only associated with redox correction but with its potential to prevent functional and structural integrity of mitochondria and mitigate the harmful of apoptosis signaling by downregulation of cytosolic Cyt-c and caspase-3, and on the other side, this extract improves AChE production, thus maintain in hippocampus cells of LCT-treated rats. This plant, following certain data, has many beneficial and healing characteristics, antioxidant and free radicals scavenging properties [24,64,67].

5. Conclusion

The results of this study concluded that long-term LCT exposure could damage hippocampus cells by altering structural and functional integrity of mitochondria, by amplifying apoptosis signaling pathway and causing cholinergic disorder. While MOE was able to prevent imbalance of redox status in hippocampus mitochondria, avoid mitochondrial membrane permeability disturbances and consequently mitigate apoptosis signaling.

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editing, RR, MK and HF. All authors read and approved the final manuscript.

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